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Visual DNA microarrays for simultaneous detection of *Ureaplasma urealyticum* and *Chlamydia trachomatis* coupled with multiplex asymmetrical PCR

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Abstract

Visual DNA microarrays, based on gold label silver stain (GLSS) and coupled with multiplex asymmetrical PCR, were developed for simultaneous, sensitive and specific detection of *Ureaplasma urealyticum* and *Chlamydia trachomatis*. 5′-end-amino-modified oligonucleotides, which were immobilized on glass surface, acted as capturing probes that were designed to bind complementary biotinylated targets DNA. The gold-conjugated streptavidins were introduced to the microarray for specific binding to biotin. The black image of microarray spots, resulting from the precipitation of silver onto nanogold particles bound to streptavidins, were used to detect biotinylated targets DNA visually or with a visible light scanner. Multiplex asymmetrical PCR of *U. urealyticum*, *C. trachomatis* and *Bacillus subtilis* (used as positive control) was performed to prepare abundant biotinylated single-stranded targets DNA, which affected detection efficiency and sensitivity of hybridization on microarray. Plenty of clinical samples of *U. urealyticum* and *C. trachomatis* from infected patients were tested using home-made DNA microarrays. For its high sensitivity, good specificity, simplicity, cheapness and speed, the present visual gene-detecting technique has potential applications in clinical fields.

Keywords: *Ureaplasma urealyticum*; *Chlamydia trachomatis*; DNA microarrays; Gold nanoparticles; Gold-conjugated streptavidins; Gold label silver stain; Multiplex asymmetrical PCR

1. Introduction

Ureaplasma urealyticum and *Chlamydia trachomatis* are the common agents of sexually transmitted diseases such as nongonococcal urethritis. Fluorescence quantitative real-time PCR has emerged as an excellent molecular detection method. However, the high-cost equipments limit its application in clinical diagnosis. Furthermore, low efficiency is the obvious deficiency. The protocol for *U. urealyticum* and *C. trachomatis* diagnostic kit is customarily used to test them separately. Clinically, *U. urealyticum* and *C. trachomatis* usually co-infect the nongonococcal urethritis patients. Thus, it is essential to develop more efficient methods for the early diagnosis of *U. urealyticum* and *C. trachomatis* infections. DNA microarrays or gene chips represent one of the main new breakthroughs in molecular analysis through miniaturization of the assay and the ability to permit monitoring a large number of genes simultaneously (Schena, 1996; Brown and Botsein, 1999). This would allow for a high-throughput detection for multiple pathogens in a large number of samples. The conventional fluorescence detection method of DNA microarrays relies on the expensive confocal scanner, which restricts its use in the research field and makes it unsuitable for the reading in a wide clinical and daily-based practice (Schena and Renu, 1998; Bowtelld, 1999). Tremendous efforts have been devoted toward development of new labeling and detecting methodologies that can provide sensitive and low-cost detection of nucleic acids (Lockhart and Winzeler, 2000; Kwok, 2001). Taton et al. (2000) reported a novel method for DNA detection based on a two-probe sandwich hybridization/nanoparticle amplification coloring technique, which showed a new colorimetric strategy to detect genes with high sensitivity. The limitation of the method was that for each application nanogold particles must be prepared with the specific probes. Alexandre et al. (2001) used gold-conjugated streptavidins as universal detection probes to replace

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the nanogold-supported DNA detection probes and proved that the sensitivity of colorimetric detection using silver precipitation was comparable to that of detection in fluorescence using a confocal scanner. At the same time, preparation of abundant biotinylated single-stranded targets DNA is the critical factor affecting detection efficiency and sensitivity of targets DNA hybridization on microarray. When the 5′-end-amino-modified capture probes hybridize with biotinylated target strands denatured from the conventional PCR products, the complementary strands compete with the capture probes resulting in the decrease of the hybridization efficiency. By the multiplex asymmetrical PCR, plenty of biotinylated single-stranded targets DNA can be prepared, which can greatly enhance the efficiency and sensitivity of hybridization and detection.

In this work, sensitive, visual, simultaneous, cheap and convenient detection of *U. urealyticum* and *C. trachomatis* is presented by the method that combines chip technology, gold label silver stain and multiplex asymmetrical PCR.

2. Materials and methods

2.1. Primers and probes

To confirm the reliability of DNA amplification and genechip detection, *Bacillus subtilis* proB gene was chosen as positive control template and *Treponema pallidum* tpp47 gene as negative control. Conserved DNA sequences of urease gene for *U. urealyticum*, trp gene for *C. trachomatis*, proB gene for *B. subtilis*, and tpp47 gene for *T. pallidum* were obtained from Genebank by DNA alignment. Primers and probes were designed and selected by Primer Premier 5.0. All the primers and probes were synthesized and modified by Huanuo Biological Science and Technology Co. Ltd. (Shanghai, China). The reverse primers were modified with biotin at their 5′-end and the probes with $H_2N(CH_2)_6$–O–(PO_3)–$(T)_{10}$ at their 5′-end. Ten T bases were inserted into the probes so as to avoid the steric hindrance affecting the immobilization on the chips and hybridization of the probes with complementary targets DNA. The sequences of the primers and probes are listed in Tables 1 and 2. The lengths of DNA fragments obtained by PCR of *U. urealyticum*, *C. trachomatis*, and *B. subtilis* were 189, 135 and 175 bp, respectively.

Table 1
The primer sequences for the *U. urealyticum*, *C. trachomatis*, and *B. subtilis* detection[a]

Locus	Primer	Size (nt)	Sequence (5′ → 3′)
U. urealyticum	U-F[b]	23	AAAGATTAACAAGATACCTTTCA
	U-R[c]	20	CAGCAAAGTTCATTGAAGCA
C. trachomatis	C-F	20	AATGCATGAGTCAGGACGAG
	C-R	20	GAAACTAAATGTGCGAGAGC
B. subtilis[d]	B-F	20	GGGAAAAACTGAAGGTGATT
	B-R	20	AGACTTGAGCCGTTATGGAT

[a] The bold base was modified with biotin at its 5′-end.
[b] F: Forward primer.
[c] R: Reverse primer.
[d] Positive control.

Table 2
The probe sequences for the *U. urealyticum*, *C. trachomatis*, *B. subtilis* and *T. pallidum* detection[a]

Locus	Probe	Size (nt)	Sequence (5′ → 3′)
U. urealyticum	U-P	24	GCTGATCGTATTGCTGGTGTTGAA
C. trachomatis	C-P	21	CCACCGATGAAGAGGCGTTAC
B. subtilis[b]	B-P	24	GCACATATATCGGTGATAAAGAGC
T. pallidum[c]	T-P	25	CCCATCTGGTCAGTCTGTCAGTCGA

[a] The bold base was modified with H_2N–$(CH_2)_6$–O–(PO_3)–$(T)_{10}$ at its 5′-end.
[b] Positive control.
[c] Negative control.

2.2. Preparation of Au colloid and gold-conjugated streptavidins

The method for preparation of Au colloid described by Grabar et al. (1995) was used in the present work. The size of the gold nanoparticles was determined by a transmission electron microscope (TEM, HITACHI H-8100, Japan) with a size range of 8–15 nm in diameter. Add 1 ml colloid gold solution, adjusted to pH 6.5 with 0.1 M K_2CO_3, to 11 tubes respectively. Subsequently, 100 μl of serial diluted streptavidins (Sigma) solution containing 5–50 μg streptavidins was added to the 10 tubes and 100 μl of super-pure water was added to the eleventh tube as blank control. The 11 tubes of mixture solution were incubated for 5 min at room temperature. Thus, different tubes containing increasing concentration of streptavidins from 5 to 50 μg in 1100 μl were prepared. After 100 μl of 10% NaCl was added to each tube and incubated for 2 h, the tubes with streptavidins concentration which could saturate nanogold particles still remained their primary color while others changed from clear red to blue. The lowest streptavidins concentration, saturating the nanogold particles and remaining its primary red, was the optimal one. The optimal streptavidins concentration was 30 μg/100 μl and the actual concentration was 33–36 μg/100 μl. Five percent bovine serum albumin (BSA) was added to the mixture of gold-conjugated streptavidins probes to a final concentration of 1%. After incubated for 15 min, the mixture was centrifuged at 20,000 rpm at 4 °C for 45 min for three times. The red precipitate was washed with 20 mM Tris–buffer (pH 6.5) including 1% BSA and 0.02% NaN_3. The precipitate was suspended in the same buffer and stored at 4 °C.

2.3. Swab samples treatment and DNA extraction

Add 1 ml sterile normal saline to the test tube containing the urethral or cervical swabs collecting from the clinical *U. urealyticum*, *C. trachomatis*, or *U. urealyticum*/*C. trachomatis* positive patients provided by Hubei Maternal and Child Health Hospital (Wuhan, China) and eddy the test tube tempestuously on the vortex agitator. After proteinase K (200 μg/μl) (Wuhan Tianyuan Biotech Co. Ltd., China) digestion in a lysis buffer (25 mM EDTA, 75 mM NaCl, 0.01% SDS), DNA was extracted from 500 μl of clinical samples with phenol–chloroform. The DNA collected after precipitation in ethanol was submitted to the PCR assay. Plasmids containing the *B. subtilis* proB gene were provided by the Laboratory of Microbial Genetics of College of